Infliximab and the TNFα system

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ABSTRACT

Infliximab, a chimeric monoclonal antibody against TNFα, is efficacious in Crohn’s disease (CD) and rheumatoid arthritis (RA). Its main mechanism of action is thought to be the induction of apoptosis. The present study evaluates in detail the effects of infliximab on the TNFα system using PB monocytes and T cells as well as lamina propria lymphocytes (LPLs) from normal individuals (NI) and patients with CD, ulcerative colitis (UC), and RA. Lymphocytes were studied in the resting state in the absence of strong stimuli that may obscure subtle findings.

Infliximab did not change the numbers of viable cells. Rather, it caused monocytes to increase their release of soluble TNFR2 which serves to neutralize TNFα, potentiating the action of infliximab. It reduced TNFR2 expression, thereby decreasing TNFα responsiveness. These changes were due to up-regulated production of TNFR2 rather than increased shedding. Infliximab did not cause rebound production of TNFα transcripts that would counteract its effects. It specifically enhanced production of IL-10 but not proinflammatory cytokines secreted by leukocytes, thereby promoting an anti-inflammatory microenvironment. In addition, infliximab caused a rise in c-Jun amino-terminal kinase (JNK) phosphorylation by monocytes. Thus, infliximab manipulates the TNFα system to promote its anti-TNFα effects.

INTRODUCTION

Infliximab is a chimeric monoclonal antibody against TNFα with a murine variable region and a human immunoglobulin constant region. It has prolonged
efficacy against rheumatoid arthritis (RA), Crohn’s disease (CD), psoriasis, and ankylosing spondylitis (35). However, it can reactivate tuberculosis and may increase the mortality in sepsis (20, 1).

Several mechanisms have been proposed to explain the actions of infliximab. First, it is a TNFα neutralizer, binding both monomeric and trimeric forms of this cytokine (32). Second, infliximab causes apoptosis of activated T cells and lamina propria lymphocytes (LPLs) (7, 11, 22, 28, 33, 36, 37). Its ability to induce apoptosis of monocytes is controversial as shown by conflicting in vitro data (22, 28). Similarly, monocyte counts decline with infliximab infusion in some studies (21, 25) but not others (28). Third, infliximab alters cytokine secretion in the serum and lamina propria, decreasing IL-1, IL-6, IL-18, and IFNγ production (2, 3, 4, 8, 10, 21, 25, 26, 28). TNF receptor (TNFR) types 1 and 2 were elevated in the serum of patients with active CD and declined with infliximab treatment (15, 25). Effects on IL-10 are controversial (2, 19, 23, 25, 34). Finally, infliximab upregulates p38 MAP kinase activity (30, 38). However, upregulation of p38 MAP kinase also occurs with IBD (39), so the relevance of this finding is uncertain.

Besides binding and neutralizing soluble TNFα, infliximab also binds membrane (m)TNFα (24, 33). Reverse signaling through mTNF has been demonstrated using peripheral blood CD4+ cells, infected T cells, and lipopolysaccharide (LPS)-stimulated monocytes (9, 5, 13, 17, 40), resulting in calcium influx and secretion of cytokines. Membrane TNFα without the soluble
form is sufficient to cause colitis and arthritis, suggesting pathogenesis through reverse signaling of mTNFα (9).

TNFR1 is the key mediator that signals soluble trimerized TNFα. Binding of TNFα to TNFR1 results in internalization of the complex and induction of the apoptotic signaling cascade. Binding of TNFα to TNFR2 results in shedding of the receptor followed by the use of overlapping signaling pathways resulting in cellular activation (16). Soluble TNFR2 is a natural TNFα inhibitor. The downstream signaling of both TNFRs includes the activation of the three major MAP kinase families--p38, ERK, and c-Jun amino-terminal kinases (JNK) (27).

The present study evaluates the effects of infliximab on monocytes, T cells, and LPLs. Monocytes, in particular, have been shown to be important mediators of RA and CD (14). The system used in this study is the culture of leukocytes in medium alone with or without infliximab, so that subtle changes, perhaps hidden by strong stimulation and resulting apoptosis, can be identified. The concentration of infliximab used, 20 ug/ml, is just above the serum trough level (31).

**METHODS**

**Isolation and culture of leukocytes**

Blood samples and intestinal mucosal specimens were obtained from normal individuals (NI) and from patients with CD, ulcerative colitis (UC), or RA after informed consent. This project was approved by the Institutional Review Board at Robert Wood Johnson Medical School. Normal small bowel was obtained from patients undergoing gastric bypass operations for morbid obesity.
Normal large bowel was obtained from patients undergoing resection for cancer, polyps, or diverticulitis. Small and large bowel specimens were obtained from patients with CD or UC undergoing surgical resection for strictures, fistulas, severe disease, or dysplasia/cancer. Unless otherwise stated, the specimens used from IBD patients were grossly involved with disease. Disease activity was determined by the CD activity index (CDAI), where values greater than 200 were considered to be active disease. The disease activity index (DAI) score was used for UC; a rating of 4 was considered to be active disease. All except two CD patients and one UC patient out of the 21 and 9, respectively, used in this study had active disease. Immunosuppressive medications were considered to be prednisone (more than 15 mg/day), 6-mercaptopurine, methotrexate or their equivalents. No patient was taking infliximab at the time of the study. Sixteen CD patients and six UC patients were on immunosuppressive medications. Patients with RA were diagnosed using the criteria of the American College of Rheumatology (36).

Peripheral blood lymphocytes (PBLs) were isolated by Ficoll density gradient centrifugation. To obtain monocytes, PBLs (1 x 10^5/0.1 ml) were incubated in wells and the nonadherent cells removed. This provided a dense layer of monocytes needed to detect cytokines optimally. T cells were obtained by recovering the nonadherent cells and negatively depleting them of CD14^+, CD20^+, and HLA-class II (DR)^+ leukocytes (antibodies from R & D Systems, Minn, MN) using magnetic beads coated with goat anti-mouse IgG (BD Biosciences, Pharmingen, San Diego, CA).
Lamina propria lymphocytes (LPLs) were obtained as described previously (12). In brief, minced intestinal mucosa was treated in a shaking water bath (37°C) with 1 mM dithiothreitol, then with 0.75 mM ethylenediamine tetra-acetic acid (both from Sigma Aldrich, St Louis, MO), and finally collagenase (Worthington Scientific, Malvern, PA). The cells from the collagenase digestion were isolated by a Percoll density gradient (Pharmacia Fine Chemicals, Piscataway, NJ). Any remaining epithelial cells were then removed by negative selection using an anti-epithelial cell antigen (BER-EP4, Dako, Glostrup, Denmark) followed by magnetic beads coated with goat anti-mouse IgG (Polysciences, Warrington, PA). Any preparation containing <80% CD45+ lymphocytes was discarded as epithelial cell contamination markedly reduces lymphocyte viability.

Cells were cultured in RPMI with 10% fetal calf serum, 1% glutamine, and 1% antibiotics/antimycotics (all from Sigma Aldrich). Cultures were supplemented with infliximab (Centocor, Malvern, PA), Z-VAD-fmk (50 μg/ml) (Sigma Aldrich), soluble TNFR2, anti-TNFR2 antibody, IL-10, or antibody neutralizing IL-10 (all from R & D Systems). The metabolic inhibitors, actinomycin D, SB203580, PD98059 (Sigma-Aldrich), or JNK inhibitor II (SP600125, Calbiochem, La Jolla, CA) were added to some cultures for 30 min and then removed prior to incubation with or without infliximab.

**Evaluation of mitochondrial activity using the MTT assay**

Monocytes (1x10^4 per microwell), T cells, or LPLs (both at 1 x 10^5 cells/0.1 ml) were incubated in the presence or absence of infliximab. After one to three
days, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma Aldrich] was added and the cells incubated for 6 hrs at 37\(^{0}\)C. Color development was initiated by the addition of dimethyl sulfoxide. The amount of yellow MTT that was reduced to purple formazan was measured spectrophotometrically at an absorbance of 550 nm. This reduction takes place only when mitochondrial dehydrogenase enzymes cleave the tetrazolium rings, a process that is directly related to the number of viable cells.

**Immunofluorescence and measurement of apoptosis**

Cells were stained by indirect immunofluorescence using antibody recognizing IL-10, TNF\(\alpha\), or TNFR2, followed by goat anti-mouse IgG (GAM) conjugated to phycoerythrin (PE) (R&D Systems). For intracytoplasmic (ic) cytokines, cells were first fixed and permeabilized with Cytofix Cytoperm (Pharmingen, San Diego, CA) before immunofluorescence staining. In other experiments, apoptosis and necrosis were determined by labeling the cells with Annexin conjugated to fluorescein isothiocyanate (FITC) along with propidium iodide (PI) (Invitrogen, Carlsbad, CA). Expression was determined by flow cytometry (Beckman Coulter FC500). The relative fluorescence intensity (RFI) is the fold-increase in staining compared to a GAM-PE control.

**Binding of radiolabelled TNF\(\alpha\) to monocytes**

PBLs (1x10\(^{6}\)/0.2 ml) were incubated for 1 hr at 25\(^{0}\)C in RPMI 1640 with 0.01 M HEPES and 1% BSA containing 100 pg/ml \([^{125}\text{I}]\)TNF\(\alpha\) (980 Ci/mmol) (Amersham Biosciences) as described previously (29). Preliminary experiments showed that higher doses of radiolabelled TNF\(\alpha\) resulted in the same amount of
specific binding indicating that the chosen dose saturated all the binding sites. The cell suspension was then layered over ice cold 20% Percoll. To make this, 100% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), composed of 9 parts of Percoll with 1 part of 10x PBS, was diluted to 20% with binding medium (RPMI 1640 with 10 mM HEPES and 1% bovine serum albumin). This tube was then centrifuged at 12,000 g for three min. The pelleted cells were incubated with infliximab (20 ug/ml) for 1 hr and then layered again over the 20% Percoll density gradient. After another centrifugation, the tubes were snap frozen in liquid nitrogen and the tips cut off. Radioactivity in the tips (bound TNFα) and that in the supernate (unbound TNFα) was recorded. The amount of specifically bound labeled peptide was calculated as the difference between total and non-specifically bound radioligand, the latter measured in the presence of 10⁻⁶ M unlabelled TNFα.

**Determination of cytokine protein and mRNA**

IL-1β, IL-2, IL-6, IL-10, IL-12, IL-18, IFNγ, and sTNFR2 were determined using ELISA (R & D Systems). TNFα mRNA levels were determined by a quantitation kit (R & D Systems). This involved the hybridization of cellular RNA with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labelled detection probes in a microplate. The hybridization products were then transferred to a streptavidin-coated microplate, and the RNA/probe hybrid was captured. Following a wash to remove unbound conjugate, color was developed, and its quantitation was proportional to the amount of gene-specific mRNA in the original samples.
**Determination of phosphorylated MAP kinase proteins**

Monocytes, T cells, and LPLs were cultured with or without infliximab for 1, 2, or 4 hrs and then fixed and permeabilized and finally stained for phosphorylated p38, ERK, or JNK followed by goat anti-rabbit conjugated to FITC. Immunofluorescence was read by flow cytometry.

**Statistical Analysis**

Pairs of data were compared using the Student’s t test or the Mann-Whitney test. For more than two groups of data, analysis of variance (ANOVA) was used with the Tukey test to compare pairs of data sets within the group.

**RESULTS**

**Infliximab does not induce significant cell death of resting mononuclear cells.**

A major mechanism of infliximab is thought to be the induction of apoptosis. Whether this occurs with resting monocytes is controversial (22, 28). To reevaluate this question, peripheral blood (PB) monocytes, T cells, or LPLs were incubated with infliximab (20 μg/ml) or medium alone, without an additional stimulus, for 18 hrs and stained by immunofluorescence for Annexin and PI (Fig. 1). Infliximab did not change the low numbers of positively-stained monocytes or T cells derived from normal individuals or from patients with CD, UC, or RA. Similarly, LPLs from normal individuals were unaffected by infliximab. However, LPLs from areas involved with CD or UC (n=3 and n=3, respectively) did show a slight increase in Annexin/PI staining in the presence of infliximab, whereas those from uninvolved tissue (n=3 and n=3 for CD and UC, respectively) did not.
To determine whether there was a significant decline in the numbers of viable cells, we cultured monocytes, T cells, or LPLs from normal individuals or patients with CD, UC, or RA with or without infliximab for up to three days (Fig. 2) and tested viability in the MTT assay. Here, the yellow tetrazolium salt (MTT) is reduced by metabolically-active cells resulting in the formation of insoluble purple formazan, the color of which can be quantitated by spectrophotometry. This assay showed that infliximab did not affect the numbers of viable cells. The slight increase in apoptosis with CD and UC LPLs did not affect the numbers of viable cells. This suggests that the action of infliximab on resting cells is not due to a decline in cell numbers.

**Infliximab reduced TNFR2 expression on monocytes.**

Monocytes, from normal individuals or from patients with CD, UC or RA, cultured in medium alone for 18 hrs, expressed TNFR2 with an RFI of 1.77 $\pm$ 0.14. This declined to 1.4 $\pm$ 0.10 with infliximab ($p<0.05$, n=12) (Fig. 3 showing results from normal individuals). The loss of TNFR2 expression likely lowers their response to TNF$\alpha$, thereby contributing to the anti-TNF$\alpha$ effect of this drug.

TNFR2 on T cells from all three patient groups (n=3 for each) and on LPLs from both normal individuals (n=3) and IBD patients (n=6) was nearly undetectable whether or not infliximab was in the culture (not shown). TNFR1 expression was low on all cells so a decline was difficult to detect.

**Infliximab increases sTNFR2 secretion by monocytes.**

The effects of infliximab may be reversed if it causes cells to reduce sTNFR2 release, a molecule that binds and neutralizes soluble TNF$\alpha$ (Fig. 4).
However, infliximab added to the culture in fact increased release of sTNFR2 by monocytes, whether derived from normal individuals (n=8) or from patients with CD, UC, or RA (n=12), further adding to the TNFα neutralization. The amounts released by T cells (n=8), in contrast, were much lower and were unaffected by infliximab. Release by normal, CD, and UC LPLs (n=5, n=7, and n=4, respectively) was undetectable with or without infliximab.

The reduction in membrane TNFR2, associated with an increase in sTNFR2, could be from increased shedding of this receptor. However, when actinomycin D was added, sTNFR2 release was greatly reduced, indicating that its rise was due to new protein synthesis (Fig. 5). To assure that this effect was not due to apoptosis and release of TNFR2 by dying cells, Z-VAD-fmk, a pan-caspase inhibitor, was added with or without infliximab. It did not affect the release of sTNFR2 (not shown).

Infliximab does not displace TNFα attached to its receptors

Perhaps free sTNFR2 increases as it is displaced from binding to TNFα by addition of infliximab. This receptor has a high “off” rate, indicating that TNFα is not tightly bound. To determine whether infliximab can unseat receptor-bound TNFα, two approaches were taken to examine soluble and cell-bound TNFα (Fig. 6). First, a known quantity of sTNFR2 was incubated with TNFα followed by infliximab, and the amount of free sTNFR2 was measured by ELISA (n=4) (Fig. 6A). When infliximab was added to sTNFR2, there was no change in the amount of free sTNFR2 measured. However, with TNFα, the amount of free sTNFR2 declined. If large amounts of infliximab (20 μg/ml) were then added to a cocktail
containing TNFα and sTNFR2, the amounts of free sTNFR2 did not change indicating that TNFα bound to sTNFR2 is not displaced by the addition of infliximab.

To look at this same question using cell-bound TNFα, PBLs were incubated with $^{125}$I- TNFα for 30 min at 4°C, then washed to remove free radiolabel (n=4)(Fig. 6B). Then, infliximab was added, and the amount of labeled TNFα bound to the cell was determined. There were no changes with the addition of infliximab indicating that the TNFα remained bound to the cell.

Infliximab markedly and specifically increases IL-10 production by monocytes and normal LPLs.

Infliximab has been shown to alter cytokine profiles in vivo in the serum of treated patients and in vitro using peripheral blood and colonic leukocytes and T cell lines (2, 3, 4, 8, 10, 15, 21, 23, 25, 26, 28, 34). To evaluate its effects on the cytokine profiles, unstimulated monocytes, T cells, and LPLs were incubated with or without infliximab for up to 3 days (Fig. 7). The concentrations of cytokines were determined by ELISA.

Infliximab augmented IL-10 production by monocytes on days 1 and 2 from both normal individuals and patients with CD, UC, or RA (n=12). However, it did not change synthesis of IL-1, IL-6, IL-12, or IL-18, indicating a selective action (n=12). Similarly, T cells demonstrated a small increase in IL-10 on day 1, but no changes in IFNγ, IL-2, or IL-6. LPLs from normal individuals, but not those from patients with CD or UC (n=6, n=5, and n=4 respectively), also increased IL-10 release with infliximab. The results using uninvolved IBD
mucosa were mixed with an increase in IL-10 production in some (n=3) but not all (n=3) experiments. IFNγ, IL-2, and IL-6 release was unaffected by infliximab using LPLs from either normal individuals or patients with CD or UC. Overall, an up-regulation of IL-10 by monocytes and LPLs without an associated increase in IFNγ and other proinflammatory cytokines contributes to an anti-inflammatory microenvironment.

**Infliximab does not affect TNFα transcription**

The actions of infliximab may be reduced if the cells respond by increasing TNFα production. To evaluate this, TNFα mRNA was measured at 4 or 18 hrs using monocytes, T cells, or LPLs from normal individuals or patients with CD in the presence or absence of infliximab (Fig. 8). The numbers of transcripts remained constant for all cell types, suggesting a lack of feedback regulation at the transcriptional level. Intracytoplasmic expression of TNFα in monocytes declined from an RFI of 1.5±0.1 with medium to 1.3±0.1 with infliximab (n=3, p=0.07). In LPLs from patients with CD, the expression changed from 2.9±0.8 with medium to 2.0±0.2 (n=4, p=not significant). These data also indicate a lack of upregulation of TNFα with infliximab.

**Infliximab increases expression of JNK**

Since infliximab has been shown to increase expression of p38 (30, 38), this, as well as other MAP kinase proteins, were measured in the presence or absence of infliximab after 2 hrs in culture (Fig. 9). There was a slight increase in p38 and ERK but a marked increase in JNK using PB monocytes from normal
individuals or patients with CD, UC, or RA (each n=3). This did not occur with T cells (each n=3) or LPLs (n=5 with CD and n=3 with UC).

To determine whether MAP kinases were involved in the production of TNFR2 or IL-10, specific inhibitors were added to the cultures and the resulting changes in cytokine expression was measured. Macrophage production of IL-10 declined with the inhibitor of p38 at 5 μg/ml, but not at lower concentrations (Fig 10). Production did not change with the inhibitor of ERK or JNK. TNFR2 release was unaffected by inhibitors of p38, ERK, or JNK (Fig. 5).

DISCUSSION

This study describes several new actions by infliximab on resting monocytes, T cells, and LPLs without an associated change in cell numbers. First, infliximab reduced surface TNFR2 expression by monocytes, limiting the TNFα-responsiveness of these cells. Second, infliximab increased TNFR2 release by monocytes, thereby contributing to their overall TNFα-neutralizing capacity. Third, it enhanced production of IL-10, particularly by monocytes and normal LPLs with a lesser up-regulation by T cells. This action was selective since infliximab did not affect synthesis of IL-1, IL-2, IL-6, IL-12, IL-18, or IFNγ. Fourth, it up-regulated phosphorylation of p38, ERK, and particularly JNK; metabolic inhibitors of p38, but not inhibitors of ERK or JNK, reduced IL-10 release. Infliximab did not trigger a compensatory rise in TNFα production that would reverse its anti-TNFα effects, nor did it displace sTNFR2 binding to TNFα. These findings did not differ whether the patient had CD, UC, or RA, suggesting that infliximab has a uniform effect regardless of the underlying condition.
The mechanism of action of infliximab on resting monocytes, T cells, or LPLs was not due to a decline in numbers of viable cells. Viable cell numbers, measured by the MTT assay, was unaffected by infliximab. Also, addition of the general caspase inhibitor had no effect on the rise in IL-10 or sTNFR2 levels. When measuring apoptosis, there was no change in the numbers of Annexin+ monocytes with exposure to infliximab. The lack of infliximab-induced apoptosis of resting monocytes agrees with one but not another study (22, 28). Although the reason for this discrepancy is unclear, it may relate to the different techniques used to isolate the monocytes: one using density gradient centrifugation (22) and the other using negative selection (28). The increase in apoptosis noted with CD and UC LPLs was not reflected in a change in the numbers of viable cells. This may be due to a small change in apoptosis not registered by the MTT assay. Alternatively, there may be proliferation of LPLs counteracting the loss of cells through apoptosis although this would likely be too small to measure.

Infliximab reduced the expression of TNFR2 on monocytes. This may decrease their response to TNFα, further promoting infliximab’s action. Membrane-bound TNFR2 is up-regulated in CD and promotes experimental colitis (18) so a decreased level of expression may be therapeutic.

Infliximab increases the amounts of TNFR2 secreted by monocytes, an action inhibited by actinomycin D. This suggests up-regulated synthesis and transport of new TNFR2 to the cell surface. Soluble TNFR2 is a known natural inhibitor of TNFα, so this action potentiates the anti-TNFα effects of infliximab.
The effects of infliximab on IL-10 release depend upon the system used. IL-10 is raised in the serum after infliximab infusion and in the cultures of Jurkat cells (23, 25). In contrast, infliximab added to LPS-stimulated monocytes reduced IL-10 synthesis, although this could be due to the associated apoptosis (34). There was a decline in the ability of mitogen-activated PBLs isolated after infliximab infusion to produce IL-10 (10); whether there was a change in the percentage of monocytes is unknown. In addition, IL-10 in cultures from biopsies was reduced if taken after infliximab treatment compared to the elevated levels found beforehand (2). Again, this could be due to the numbers and types of leukocytes in the biopsies. By using unstimulated monocytes, the present study showed that the increased IL-10 with infliximab was not due to changes in cell number or apoptosis. Infliximab also increases IL-10 production by LPLs from normal individuals but not from those derived from patients with CD or UC.

The present study shows that the monocyte-derived cytokines—IL-1, IL-6, IL-12, and IL-18—were unchanged with infliximab, indicating that the increase in IL-10 is selective. Amounts of certain cytokines—IL-1, IL-6, IL-18, and IFNγ—have been shown in the literature to be decreased in the serum and/or in culture medium of circulating or intestinal leukocytes (2, 3, 4, 8, 10, 21, 25, 26, 28). Some of these changes may be due to apoptosis of the cells or to an overall improvement in disease. The findings also depend upon the system studied. IFNγ production, for example, has been found to be reduced by infliximab in colonic T cell cultures and unchanged in cultures of Jurkat cells (3, 15).
In the present report, JNK phosphorylation increased in monocyte cultures exposed to infliximab. This event occurred in Jurkat cells and apoptosis of these cells was inhibited by SP600125, an anthrapyrazolone inhibitor of JNK (23).

Monocytes had measurable responses to infliximab, whereas LPLs did not. Infliximab down-regulated expression of TNFR2 in monocytes, whereas this marker remained undetectable in LPLs. Infliximab increased sTNFR2 release by monocytes, but this, again, was undetectable in LPL cultures. Infliximab boosted IL-10 production by monocytes, T cells, and normal LPLs, but not by CD or UC LPLs. Infliximab raised phosphorylated JNK levels in monocytes but not in LPLs. Monocytes have been shown to be important mediators of RA and CD (14). While they are present in mucosa, many are not retrieved by cell separation techniques. Therefore, infliximab may exert its effects on mucosal macrophages although these effects are not demonstrated by isolated LPLs.

Since most patients undergoing surgical resection for IBD have active disease, there were too few patients with inactive disease in this study to make a determination as to whether disease activity influences the results. Similarly, most patients were on immunosuppressive medications so the influence of these medications could not be assessed.

The present findings occur in the context of resting lymphocytes which are likely to be the steady state in vivo. Infliximab does not increase TNFα production, but rather diminishes TNFα responsiveness through a decline in TNFR2 expression and an increase in release of the TNFα-neutralizing TNFR2. These findings contradict those showing an increase in TNFR2 release with
active IBD that decreases with infliximab (15, 25). Such findings, however, may not be a direct effect of infliximab but rather a reflection of the overall improvement in disease activity. Infliximab has excellent therapeutic success that lasts weeks probably due to the lack of a mechanism to reverse its effects. However, there is a high relapse rate after termination of treatment. This could be due to the lack of significant apoptosis. The potentiation of the TNFα inhibition by infliximab highlights the importance of this cytokine in the pathophysiology of IBD and RA and suggests a common mechanism of action in these diseases. It would be of interest to determine whether the response to infliximab correlates with changes in this TNFα system.

In summary, infliximab has some critical effects on the TNFα system. It is essential that it not reverse its effects by its actions on TNFα production, TNFα neutralization, or TNFα responsiveness. And, in fact, it does not affect TNFα production, promotes TNFα neutralization by the release of sTNFR2, and reduces TNFα responsiveness by a decrease in TNFR2 expression. Infliximab increases IL-10 production contributing to an anti-inflammatory microenvironment. Studying leukocytes in the resting state avoids the confounding apoptosis and may reflect their steady state.

References


Figure Legends

**Fig. 1:** Monocytes, T cells, and LPLs were cultured for 18 hrs with or without infliximab (20 μg/ml) and the numbers of apoptotic and necrotic cells determined by staining with annexin and PI. The percentages of cells in each quadrant are depicted. The results with monocytes and T cells were the same for normal individuals and patients with CD, UC, or RA. LPLs differed depending upon the source so results from normal individuals and patients with CD (from inflamed areas) are shown.

**Fig. 2:** Monocytes, T cells, or LPLs were cultured for up to 3 days with infliximab (20 μg/ml) or medium alone. Cell viability was measured by the MTT assay and the results expressed as optical density (OD). Since monocytes and T cells acted the same whether derived from normal individuals or from patients with CD, UC, or RA, the results were combined. LPLs also acted the same regardless of the source, but the data was segregated so as to emphasize the lack of difference whether cells were derived from normal individuals or from patients with CD or UC.

**Fig 3:** Monocytes and T cells from normal individuals were cultured with or without infliximab (20 ug/ml) for 18 hrs and then directly stained for TNFR2 surface expression.

**Fig. 4:** Monocytes and T cells were cultured with or without infliximab (20 ug/ml) for one to three days, and sTNFR2 was quantitated in the culture medium. Results from normal individuals and patients with CD, UC, or RA were
combined as there were no differences among the groups. Significant differences were computed using the paired Student’s t test.

**Fig. 5:** Monocytes from normal individuals or patients with CD (results combined) were cultured with metabolic inhibitors, then tested for sTNFR2 release by ELISA. Actinomycin D is an inhibitor of RNA synthesis; SB203580 is an inhibitor of p38; PD98059 is an inhibitor of ERK, while SP600125 is an inhibitor of JNK. The asterisks indicate the value that is significantly different from “none” (p<0.01).

**Fig. 6A:** Recombinant sTNFR2 (200 pg/ml) was incubated with TNFα (200 pg/ml), followed by infliximab (20 μg/ml), and the amount of free sTNFR2 measured by ELISA.

**Fig. 6B:** PBLs were saturated with radiolabelled-TNFα (100 pg/ml), then exposed to infliximab (20 μg/ml). The binding of labeled TNFα was calculated by the amount of radioactivity found on the PBLs.

**Fig. 7:** Monocytes, T cells, or LPLs were cultured for up to 3 days in the presence or absence of infliximab (20 μg/ml). The supernates were tested by ELISA for IL-10. Results from normal individuals and patients with CD, UC, and RA were combined for monocytes and T cells. Results for LPLs were segregated into those from normal or IBD mucosa. Significant differences were computed using the paired Student’s t test.

**Fig. 8:** Monocytes, T cells, or LPLs were cultured for 18 hrs with or without infliximab and the amount of TNFα transcripts determined. Values from normal individuals and patients with CD were pooled as the results were the same.
**Fig. 9:** In this representative of 4 experiments, T cells (A) or monocytes (B) were cultured with or without infliximab (20 μg/ml), and ic p38, ERK, or JNK determined by immunofluorescence after 2 hrs. The lighter line represents the results in medium alone which was equivalent to the control containing GAM-FITC alone. The RFI refers to the expression with infliximab.

**Fig. 10:** Monocytes were cultured with or without infliximab in the presence or absence of inhibitors. Actinomycin D is an inhibitor of RNA synthesis; SB203580 is an inhibitor of p38; PD98059 is an inhibitor of ERK, while SP600125 is an inhibitor of JNK. IL-10 production was determined by ELISA. The asterisks mark those values significantly less than “none”: **=p<0.01; ***=p<0.001.
Fig. 1:
Normal monocytes with medium alone

Normal monocytes with infliximab

Normal T cells with medium alone

Normal T cells with infliximab
Normal LPLs with medium alone

Normal LPLs with infliximab

CD LPLs with medium alone

CD LPLs with infliximab
Fig. 2:

**monocytes**

- Day 1: Viable cells, OD 0.55 ± 0.05
- Day 2: Viable cells, OD 0.63 ± 0.06
- Day 3: Viable cells, OD 0.58 ± 0.04

**T cells**

- Day 1: Viable cells, OD 0.42 ± 0.03
- Day 2: Viable cells, OD 0.50 ± 0.04
- Day 3: Viable cells, OD 0.55 ± 0.05

**Normal LPLs**

- Day 1: Viable cells, OD 0.48 ± 0.03
- Day 2: Viable cells, OD 0.55 ± 0.04
- Day 3: Viable cells, OD 0.52 ± 0.05

**CD LPLs**

- Day 1: Viable cells, OD 0.50 ± 0.03
- Day 2: Viable cells, OD 0.53 ± 0.04
- Day 3: Viable cells, OD 0.56 ± 0.05
Fig. 3:

**monocytes**

**T cells**

- IgG control
- TNFR2 with medium
- TNFR2 with infliximab

Fig. 4:

**monocytes: sTNF-R2**

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**T cells: sTNF-R2**

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<td>750</td>
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<tr>
<td>infliximab</td>
<td>500</td>
<td>750</td>
<td>1000</td>
</tr>
</tbody>
</table>

p<0.05
**Fig. 5:**

![Graph showing TNFR2 production](image)

**Fig. 6A:**

![Graph showing TNF and Infliximab effects](image)
Fig. 6B:

![Graph showing binding of radiolabelled-TNF](image)

No infliximab         Infliximab

NS

Fig. 7:

![Graph showing IL-10 levels in monocytes and T cells](image)

monocytes: IL-10

T cells: IL-10

p<0.05  p<0.05  p<0.05
Fig. 8:

NI LPL: IL-10

IBD LPL: IL-10
Fig. 9:

A.

- p38
  - RFI = 1.1
- ERK
  - RFI = 0.9
- JNK
  - RFI = 1.5

B.

- p38
  - RFI = 1.4
- ERK
  - RFI = 1.4
- JNK
  - RFI = 2.2

Number of events

Mean channel number
Fig. 10:

IL10 production, pg/ml

Inhibitor, µg/ml

none    Act D           SB             PD            SP
5    1    .2    5   1   .2   5    1   .2  5     1   .2